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XYLANASE PRODUCTION UNDER SUBMERGED FERMENTATION BY ISOLATED *BACILLUS TEQUILENSIS* STRAIN AND ITS POTENTIAL APPLICATION IN ANIMAL FEED

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ABSTRACT

Poultry feed mainly contains large amount of Non-starch polysaccharides (NSP), which decrease energy utilization and absorption of other nutrients leading to depressed growth in broiler chicken. A xylanase has been produced from newly isolated strain *Bacillus tequilensis* under submerged fermentation in Horikoshi medium supplemented with wheat bran (0.5%), pH 8 and at 37^{o}C. After optimization of various production parameters, xylanase production was increased 3 fold. The produced xylanase is stable in neutral to alkaline pH region upto 42^{o}C. The suitability of this xylanase for use in animal feed was investigated. A xylanase dose of 500 µl enzyme dosage with 0.5g of poultry feed exhibited optimum release of reducing sugar and decrease in digesta viscosity at pH 6, after 21h of treatment. It was observed that, 87 % reducing sugar was released and viscosity decreases above 60 % as compared to control. These results indicated that a xylanase isolated from this strain and produced under given optimized conditions has great application in animal feed and is effective in releasing sugars from the feed aiding in the digestibility of feed.

KEYWORDS: Xylanase, Animal Feed, *Bacillus Tequilensis*, Poultry Feed Digesta Viscosity, Hemicelluloses

INTRODUCTION

Poultry feed majorly contains considerable amount of Non-starch polysaccharides (NSP) and phytates. These components are known for reducing the energy utilization, protein digestion and decreasing the absorption of other nutrients. The ingestion of soluble NSP like β-glucans increased the digesta viscosity and depressed the growth rate in broiler chicken (Bharthidashan *et al.*, 2009). Since, Xylan is the major compound found in the hemicellulose fraction of hardwood plant tissue and is located predominantly in the secondary cell walls of angiosperm and gymnosperms, there is a need for enzymes which can degrade xylan and improve digestibility of feed. Xylan consists of homopolymeric backbone of 1, 4- linked β-D-xylopyronase units (Annamalai *et al*., 2009). The plant cell wall is a composite material in which cellulose; hemicellulose (mainly xylan) and lignin are closely associated (Bisson *et al.*, 2002). Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%) - a group of carbohydrates in which xylan forms the major class- and lignin (20-30%) (Subramaniyan *et al.*, 2002*)*. According to their origin, xylans of hardwoods, softwoods, or grasses have different substituent that is covalently linked to the backbone (Puls *et al*., 1989). These substituents may include O-acetyl, α-L-arabinofuranosyl, α-D-glucopyranosyluronic acid and 4-o-methyl-α-D glucopyranosyluronic acid. Birchwood xylan, for instance, can be composed of up to 35% o-acetyl-4-O-metylglucorono-xylan (Timell, 1967). Xylanases are groups of enzymes that depolymerize xylan molecules into xylose units used by microbial populations as a

primary carbon source (Nath and Rao, 2001). The enzymatic degradation of xylan to xylose requires the catalysis of both endo-xylanase (EC 3.2.1.8) and β- xylosidase (EC 3.2.1.37) (Faulet *et al.,* 2005). The xylan-degrading system include endo-1, 4- xylanases (EC 3.2.1.8), which release long and short xylooligosaccharides, or those that only attack longer chains and b-D-xylosidase (1, 4-β -xylan xylohidrolase; EC 3.2.1.3.7), which remove D-xylose residues from short xylo-oligosaccharides (Knob and Caramona, 2008). Application of xylanases in biotechnology include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels (Battan *et al.,* 2007). Not much literature has been reported on the high-level production and process economy of utilizing xylanolytic enzymes. Wide scale industrial applications of xylanase require their cost-effective production to make the process economically viable. The strains reported in already published work for the commercial production of xylanases include; *Streptomyces albus* and *streptomyces chromofuscus* (Riffat *et al*., 2005), *Streptomyces lividans* (Ragauskas *et al*., 1994), *Tricoderma reesi* (Tenkanen *et al*., 1992), *Bacillus pumilus* (Battan *et al*., 2007) and *Bacillus subtilis* (Saleeem and Akhtar, 2002) but the production parameters used were not very cost effective. Thus, the aim of our studies was to reduce the cost of xylanase production by optimizing the fermentation medium and to study its application in animal feed industry. In the present investigation, we report high level production of a thermostable xylanase using agro-residues in submerged fermentation from a newly isolated strain of *Bacillus tequilensis*, that has been potentially applied in the poultry feed for enhancing the digestibility of the NSP present in feed.

MATERIAL AND METHODS

Microorganism

Bacillus tequilensis was isolated from soil sample collected from garbage dump sites using xylan agar medium at the temperature of 37°C. Its ability to produce xylanase was qualitatively confirmed when it formed digestion halos on birchwood xylan agar plates on treatment with 1% Congo red followed by washing with 1 M NaCl. The organism was identified as *B. tequilensis* from the Institute of Microbial Technology (IMTECH), Chandigarh, India on the basis of its morphological, physiological and biochemical characteristics. The culture was maintained and stored at 4 °C on nutrient agar medium.

Xylanase Production under Submerged Fermentation

The enzyme production was studied in Erlenmeyer flask (250ml) containing Horikoshi media having 5% yeast extract powder, 5% peptone, 1% KH_2PO_4, 1% K_2HPO_4, O.2% magnesium sulphate, and 0.5% wheat bran. The flask was inoculated with 1ml (O.D~0.5) 18h old culture and then incubated for 24 h in shaker incubator at 200 rpm. Cell broth was centrifuged at 10,000rpm at 4ºC for 20 minutes. The supernatant is then treated as crude enzyme extract. Supernatant containing crude enzyme was collected and stored at 4°C.

Enzyme Assay

Xylanase activity was determined by measuring the release of reducing sugar during enzyme substrate reaction using Miller's method (Miller, 1959). Birchwood xylan was used as a substrate for xylanase. The reaction mixture of assay contains 490µl of 0.1 % of substrate prepared in 0.1M phosphate buffer (pH=6), and 10 µl of appropriately diluted enzyme incubated for 10 min at 40° C in water bath. The reaction was terminated by addition of 1.5 ml 3-5, dinitrosalicyclic acid (DNSA) reagent. This was followed by boiling the reaction mixture for 15 minutes in a boiling water bath and thereafter, allowing it to cool to room temperature. The color intensity was detected at 540 nm against enzyme blank (490 µl of

buffer+10 µl of enzyme) and substrate blank (490 µl of substrate+ 10 µl of buffer) in spectrophotometer. The concentration of glucose released by enzyme was determined by comparing against standard curve constructed in similar way. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol of reducing sugar from the substrate in 1 min under standard assay conditions.

Parametric Optimization of Xylanase Production

- **Incubation Period:** Submerged fermentation was carried out for 20 to 40h.
- **Temperature:** Xylanase production at temperature ranging from 20°C to 47°C.
- **pH:** Horikoshi medium of pH ranging from 5-10 were used for xylanase production.
- **Carbon Source:** Different carbon sources viz maltose, fructose, lactose, glucose, sucrose, glycerol and starch were tested separately at 2 % concentration as sole carbon source for xylanase production

After optimization of these parameters, xylanase production was carried out under optimized fermentation conditions for maximum yield of the enzyme to be applied in animal feed.

Optimization of Enzyme Dosage and Other Parameters for Animal Feed

The optimization of pH, enzyme dosage and retention time for poultry feed was carried out by treating 0.5g of moistened poultry feed with varying dosage of 100 to 500 µl for variable time interval starting from 15 to 24h at different pH ranges (5 to 7) at 37°C. The viscosity was determined by directly observing the absorbance at 540 nm with the help of colorimeter and the reducing sugar was observed by Miller's method (Miller, 1959).

RESULTS AND DISCUSSIONS

Microbial Strain and its Growth Conditions

The isolated bacterial strain is a Gram positive, moderate thermophile with minimum, optimum and maximum temperature for growth at 25 °C, 37 °C and 42 °C, respectively. The strain is an alkalophile being capable of growing at pH values up to 10.0.

Xylanase Production under Submerged Fermentation

Effect of different incubation period on xylanase production using wheat bran as substrate under submerged fermentation condition by *Bacillius tequilensis*, was tested at different time intervals. The enzyme production started after 20h and was determined after every 4h of incubation and showed maximum production at 32h (Figure 1). The effect of temperature on xylanase production by *Bacillus tequilensis* was examined at various temperature ranges viz 20°C to 47°C for 32 h. The growth of strain was recorded from 25° C to 42° C and it did not grow at temperature above and below this range. The results showed maximum xylanase production (49 IU/ml) at 37°C (Figure 2). Some *Bacillus* sp grow at temperature ranging from 25°C to 55 °C and an optimal temperature of 40°C was reported (Azeri *et al*., 2010). The fermentation temperature of 37°C has also been reported optimum for xylanase production by *Streptomyces cuspidosporous* (Maheshwari and Chandra, 2000), *B. circulans* Teri 42 (Quershy *et al.*, 2002) and *Asperigillus nidulans* kk-99 (Taneja *et al.*, 2002). The bacterium did not produce any xylanase when grown in medium of pH 4 and xylanase production started in a medium of pH 5 and results showed maximum production (66.12 IU/ml), at pH 8.0 in previously optimized conditions (Figure 3). Xylanase production after pH 10 shows decrease in enzyme activity.

Similarly *Enterobacter* sp. showed optimum xylanase activity at pH 8 (Sharma *et al*., 2009). Xylanase production from *B. circulans* Ab16 (Dhillon *et al*., 2000) and *B. pumilus* (Battan *et al.*, 2007) has also been reported at pH 8.0.

Different carbon sources were tested to determine the ones best suited for xylanase production. The effect of 6 different carbon sources viz. sucrose, fructose, lactose, glycerol, starch and maltose at 2% concentration, introduced into the modified Horikoshi medium for xylanase production by *Bacillus tequilensis* under submerged fermentation were studied. Among these sources, fructose supplemented medium had attained the maximum production at 74.23423 IU/ml (Figure 4). Ahmad *et al*., (2009) studies, favours the results of Comacho and Agcular, (2003) that when wheat bran was used as carbon source for the growth of *Aspergillus* sp., maximum enzyme activity was observed at 72 h of fermentation.

Further the xylanase production was carried out under optimized conditions (pH, temperature and incubation time), supplemented with fructose as carbon source that resulted in 3.34 fold increase in enzyme production as compared to the production under un-optimized conditions.

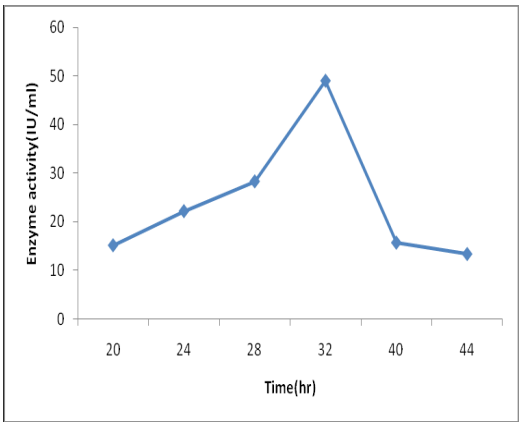

Figure 1: Effect of Incubation Time on Xylanase Production

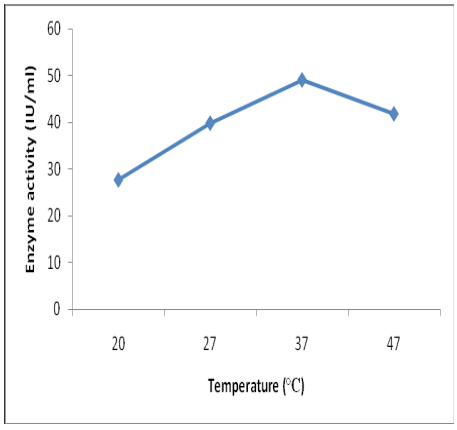

Figure 2: Effect of Temperature on Xylanase Production

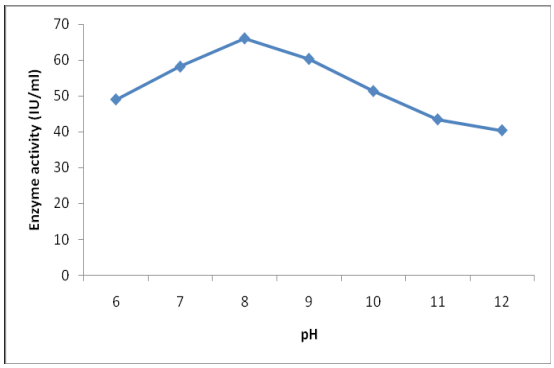

Figure 3: Effect of Ph on Xylanase Production

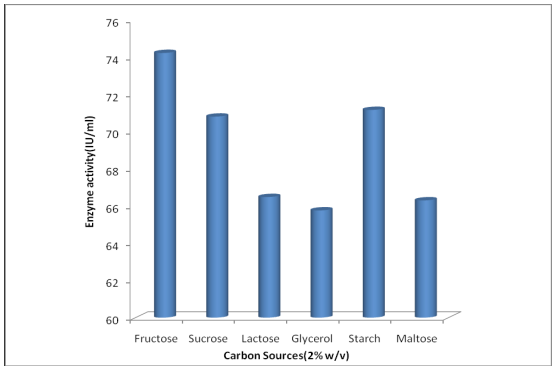

Figure 4: Effect of Various Carbon Sources on Xylanase Production

Optimization of Various Parameters for Xylanase Used in Poultry Feed

The xylanase produced by *Bacillus tequilensis* reported good activity, which promoted us to test the suitability of enzyme in poultry feed. To obtain best results from enzymatic use; pH conditions, enzyme dosage and treatment time were optimized. The results show maximum reducing sugar is released at pH 6 as compared to pH 7 and pH8. (Table 1). Evaluation of an enzyme with poultry feed showed that, increasing the level of enzyme dosage from 100 µl to 500 µl

enhanced the release of reducing sugars (Table 2) and since, the time taken after 21 h did not show any improvement in reducing sugar released, therefore only 21 h treatment time was taken (Table 3). The maximum reducing sugar released was found to be 10 mg/ml; at pH 6 with 500 µl enzyme dosage, at 21 h of treatment time. Also, there was 62% decrease in viscosity of the poultry feed treated with the partially purified enzyme as compared with the control. In this experiment 87 % reducing sugar was released, which showed that xylanase addition improves efficiency, increase nutritive value and improves the growth performance in same amount of feed as when compared to un-treated feed samples.

CONCLUSIONS

The Xylanase produced under given conditions, gave better performance in poultry feed at low cost with eco friendly nature, hence can be employed in poultry feed industry. The finding of the study reveals that the use of agro-based residues as substrate for microbial growth is possible, with good perspectives for scaling up. Moreover, results obtained in this work point to the strong necessity of employing more effort on isolating strains which requires cheaper and cost effective production parameters and keep on exploring the microbial diversity on earth to improve the standard of living.

Table 1: Effect of Ph on Poultry Feed

Ph	Abosobance (Control) OD	Absorbance (With Enzyme) OD	Reducing Sugar Released (Control) Mg/Ml	Reducing Sugar Released (Mg/Ml)
6	0.70	0.48	0.44	6.16
7	0.72	0.17	0.46	3.08
8	0.74	0.72	0.49	1.76

Table 2: Effect of Enzyme Dosage on Poultry Feed

Enzyme Dosage (µl)	Abosobance (Control) OD	Absorbance (with Enzyme) OD	Reducing Sugar Released (Control) mg/ml	Reducing Sugar Released (mg/ml)
100	0.69	0.4	0.38	2.64
200	0.70	0.29	0.42	6.16
300	0.71	0.24	0.42	7.04
400	0.72	0.20	0.43	8.36
500	0.74	0.10	0.43	9.05

Table 3: Effect of Treatment Time on Poultry Feed

Incubation Time (h)	Abosobance (Control) OD	Absorbance (with Enzyme) OD	Reducing Sugar Released (Control) mg/ml	Reducing Sugar Released (mg/ml)
15	0.70	0.34	0.40	7.04
18	0.71	0.26	0.41	7.48
21	0.72	0.10	0.43	10
24	0.74	0.12	0.44	9.06

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